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Authors
Fitzsimmons, Liam F
Liu, Lin
Kant, Sashi
et al.

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SpoT Induces Intracellular *Salmonella* Virulence Programs in the Phagosome

Liam F. Fitzsimmons,[a] Lin Liu,[a] Sashi Kant,[a] Ju-Sim Kim,[a] James K. Till,[a] Jessica Jones-Carson,[a] Steffen Porwollik,[b] Michael McClelland,[b] Andres Vazquez-Torres[a,c]

[a]University of Colorado School of Medicine, Department of Immunology and Microbiology, Aurora, Colorado, USA
[b]University of California Irvine, School of Medicine, Department of Microbiology and Molecular Genetics, Irvine, California, USA
[c]Veterans Affairs, Eastern Colorado Health Care System, Denver, Colorado, USA

ABSTRACT Guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), together named (p)ppGpp, regulate diverse aspects of *Salmonella* pathogenesis, including synthesis of nutrients, resistance to inflammatory mediators, and expression of secretion systems. In *Salmonella*, these nucleotide alarmones are generated by the synthetase activities of RelA and SpoT proteins. In addition, the (p)ppGpp hydrolase activity of the bifunctional SpoT protein is essential to preserve cell viability. The contribution of SpoT to physiology and pathogenesis has proven elusive in organisms such as *Salmonella*, because the hydrolytic activity of this RelA and SpoT homologue (RSH) is vital to prevent inhibitory effects of (p)ppGpp produced by a functional RelA. Here, we describe the biochemical and functional characterization of a *spoT*-Δ*ctd* mutant *Salmonella* strain encoding a SpoT protein that lacks the C-terminal regulatory elements collectively referred to as "ctd." *Salmonella* expressing the *spoT*-Δ*ctd* variant hydrolyzes (p)ppGpp with similar kinetics to those of wild-type bacteria, but it is defective at synthesizing (p)ppGpp in response to acidic pH. *Salmonella spoT*-Δ*ctd* mutants have virtually normal adaptations to nutritional, nitrosative, and oxidative stresses, but poorly induce metal cation uptake systems and *Salmonella* pathogenicity island 2 (SPI-2) genes in response to the acidic pH of the phagosome. Importantly, *spoT*-Δ*ctd* mutant *Salmonella* replicates poorly intracellularly and is attenuated in a murine model of acute salmonellosis. Collectively, these investigations indicate that (p)ppGpp synthesized by SpoT serves a unique function in the adaptation of *Salmonella* to the intracellular environment of host phagocytes that cannot be compensated by the presence of a functional RelA.

IMPORTANCE Pathogenic bacteria experience nutritional challenges during colonization and infection of mammalian hosts. Binding of the alarmone nucleotide guanosine tetraphosphate (ppGpp) to RNA polymerase coordinates metabolic adaptations and virulence gene transcription, increasing the fitness of diverse Gram-positive and Gram-negative bacteria as well as that of actinomycetes. Gammaproteobacteria such as *Salmonella* synthesize ppGpp by the combined activities of the closely related RelA and SpoT synthetases. Due to its profound inhibitory effects on growth, ppGpp must be removed; in *Salmonella*, this process is catalyzed by the vital hydrolytic activity of the bifunctional SpoT protein. Because SpoT hydrolase activity is essential in cells expressing a functional RelA, we have a very limited understanding of unique roles these two synthetases may assume during interactions of bacterial pathogens with their hosts. We describe here a SpoT truncation mutant that lacks ppGpp synthetase activity and all C-terminal regulatory domains but retains excellent hydrolase activity. Our studies of this mutant reveal that SpoT uniquely senses the acidification of phagosomes, inducing virulence programs that increase *Salmonella* fitness in an acute model of infection. Our investigations indicate that the coexistence of RelA/

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Address correspondence to Andres Vazquez-Torres, Andres.Vazquez-Torres@cuanschutz.edu.

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SpoT homologues in a bacterial cell is driven by the need to mount a stringent response to a myriad of physiological and host-specific signatures.

KEYWORDS *Salmonella*, bacterial pathogenesis, genetics, innate immunity, macrophages, stringent response, transposons

The alarmones guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), together named (p)ppGpp, are synthesized in the adaptation of nearly all bacterial species to nutritional starvation during a program commonly known as the stringent response (1). The stringent response is characterized by the transcriptional repression of rRNA, tRNA, and ribosomal protein genes (2, 3), and the concomitant activation of amino acid biosynthetic genes (1). Guanosine tetraphosphate also promotes the expression of alternative sigma factors, such as σ^S, and their regulons (4–6). Bacteria unable to mount the stringent response are amino acid auxotrophs, cannot adapt to nutrient downshifts, and are highly sensitive to the antimicrobial activity associated with oxidative, nitrosative, or acid stress (7–10).

Gammaproteobacteria such as *Salmonella* express RelA and SpoT homologues (RSH), which synthesize pppGpp or ppGpp through the transfer of a pyrophosphate moiety from ATP to the 3′ –OH group of GTP or GDP, respectively (11). Highly conserved RSH proteins are roughly 80 kDa in size and are expressed at low levels in most bacteria. In betaproteobacteria and gammaproteobacteria, the *relA* and *spoT* paralogs originated by duplication and have since diverged for specialized roles (12). RelA is activated in response to amino acid shortages, whereas SpoT synthesizes (p)ppGpp in response to the intracellular depletion of iron, phosphate, nitrogen, or fatty acids (13–17). In addition to its weak synthetic activity, SpoT is endowed with a strong and predominant (p)ppGpp phosphatase activity (18). The N terminus of RSH enzymes contains both (p)ppGpp-hydrolytic and (p)ppGpp-synthetic domains (HD and SYN, respectively) but, due to steric hindrance, HD or SYN activities are mutually exclusive at any given time in bifunctional enzymes such as SpoT (19). Interactions of domains in the C terminus with the HD and SYN domains influence which active site is formed (17). The RSH C terminus harbors three regulatory elements named the TGS (ThrRS, GTPase, and SpoT), INT (intermediate), and ACT (aspartate kinase, chorismate mutase, and TyrA) domains (Fig. 1). TGS and ACT regulatory domains, which were discovered in metabolic enzymes that do not synthesize or degrade (p)ppGpp, regulate enzymatic activity via allosteric binding to metabolites (12, 20). Although it is unclear whether the TGS and ACT domains of RSH homologues bind small molecules, it is apparent that the TGS domain of *Escherichia coli* SpoT facilitates protein-protein interactions. Specifically, the TGS domain mediates interactions of SpoT with acyl-carrier protein, thus inducing (p)ppGpp synthesis in response to fatty acid starvation (17). In addition, the SpoT TGS domain interacts with ObgE, a GTPase that regulates ribosome biogenesis (21, 22). The INT domain, which links the TGS and ACT domains, is highly conserved among RSH homologues and contains stretches of α-helices interrupted by short, intrinsically disordered segments (23). The *E. coli* RelA INT domain is responsible for binding to the ribosome (24), while the INT domain of *Mycobacterium smegmatis* Rel protein binds (p)ppGpp (25).

In most gammaproteobacteria species, Δ*spoT* mutants are not viable in the presence of functional *relA* alleles. This observation has led to the widely accepted idea that (p)ppGpp-hydrolysis is essential in (p)ppGpp-producing gammaproteobacteria (1). The inability to generate stable *spoT* deletion mutants has substantially hampered the study of *spoT* in bacterial physiology and pathogenesis. Compared to Δ*relA* isogenic controls, Δ*relA* Δ*spoT* mutant *Salmonella* is much more attenuated in murine models of salmonellosis (26), suggesting that (p)ppGpp synthesized by SpoT assumes an important role in the regulation of *Salmonella* virulence in mice. However, it is also plausible that RelA or SpoT each responds to distinct stimuli and synthesizes (p)ppGpp to regulate specific aspects of *Salmonella* virulence programs. In support of this view, our recent investigations have identified unique roles for RelA in the antinitrosative defenses of *Salmo-*

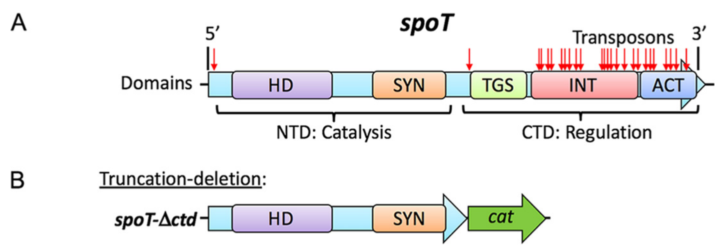

FIG 1 Diagram of the *Salmonella spoT* gene. (A) The 5′ end encodes (p)ppGpp-hydrolytic (HD) and (p)ppGpp-synthetic (SYN) domains, whereas the 3′ end encodes regulatory regions, including the (ThrRS, GTPase, and SpoT), INT (intermediate), and ACT (aspartate kinase, chorismate mutase, and TyrA) domains. Location of transposon insertion mutations in the *spoT* gene identified in our transposon library are indicated with red arrows. (B) Representation of the *spoT*-Δ*ctd* truncation allele, including the inserted chloramphenicol acetyltransferase *cat* gene.

nella (10), raising the intriguing possibility that SpoT may also adopt dedicated functions in the adaptation of *Salmonella* to specific stresses. The investigations here indicate that SpoT-derived (p)ppGpp plays a decisive role in the early activation of transcription of *Salmonella* pathogenicity island 2 (SPI-2) genes and metal cofactor acquisition genes in response to acidic cues encountered by *Salmonella* in the host cell phagosome.

RESULTS

Transposon mutagenesis identifies unique roles for *spoT* in the intracellular fitness of *Salmonella*. We examined the growth of a *Salmonella enterica* serovar Typhimurium barcoded transposon (Tn) library with ~37,353 unique mutants in J774 macrophage-like cells. This screen showed critical roles for purine and pyrimidine biosynthesis, lipopolysaccharide biosynthesis, lipid III, itaconate, and *Salmonella* pathogenicity island 2 genes. We also found that the PhoPQ two-component system, DnaJ and DksA, promotes intracellular growth of *Salmonella* (27, 28). *De novo* nucleotide and amino acid biosynthesis and uptake genes were important for growth of *Salmonella* in J774 cells (Fig. 2; see Fig. S1 and S2 in the supplemental material). The Tn screen also showed that integrations into the *spoT* gene, but not into *relA*, reduced the intracellular fitness of *Salmonella* in J774 cells. The mere existence of transposons in the *spoT* gene was surprising to us because null *spoT* alleles should not be viable. Nevertheless, the

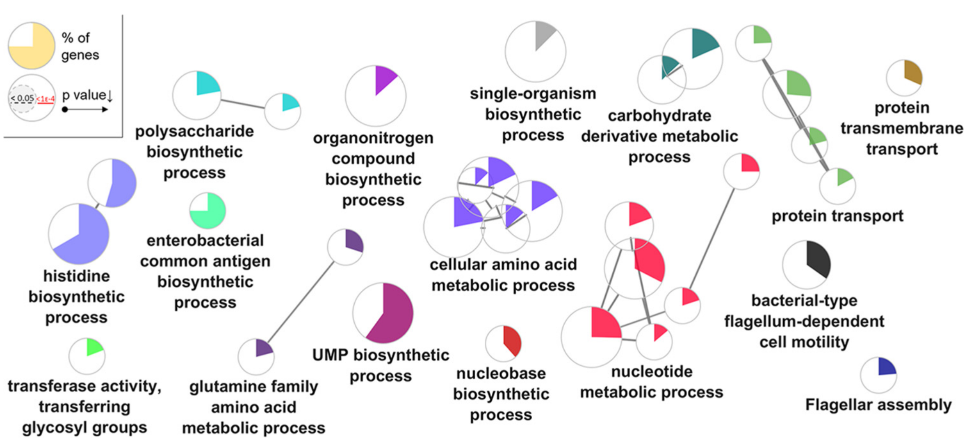

FIG 2 Genetic determinants of *Salmonella* survival in macrophages. To identify candidate *Salmonella* processes necessary for macrophage survival, negatively selected genes identified by transposon mutagenesis were categorized via pathway analysis. Significant pathways were visualized by ClueGO in Cytoscape. This analysis identifies important roles for nucleotide and histidine biosynthesis for optimal survival of *Salmonella* in J774 cells. Circle size is inversely proportionate to the *P* value, while shading is directly proportionate to the percentage of genes represented by the data for any given term. Altogether, this analysis provides a blueprint for pathways whose regulation is critical for intracellular survival of *Salmonella*. A detailed account of genes negatively selected within J774 cells can be seen in Fig. S2 in the supplemental material.

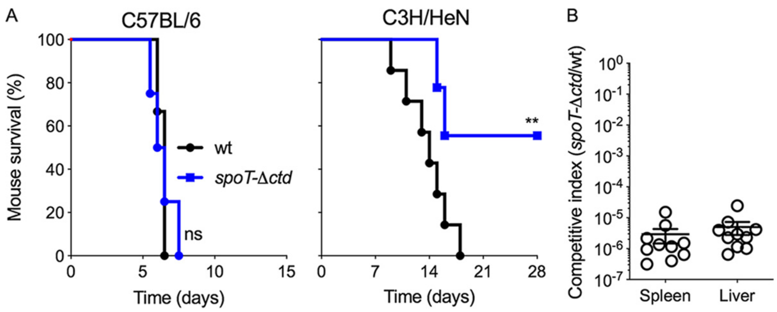

FIG 3 Virulence of *spoT*-Δ*ctd* mutant *Salmonella*. (A) Survival of C57BL/6 or C3H/HeN mice orally infected with 5×10^6 or 10^7 CFU of *Salmonella*, respectively ($N = 7$ or 9 per group). Log rank analysis; ns, not significant; **, $P < 0.01$. (B) Competitive index of wild-type and *spoT*-Δ*ctd* mutant *Salmonella* inoculated orally (p.o.) into C3H/HeN mice. The mice were euthanized at the time when they started to show signs of disease (13 to 17 days after inoculation). $N = 10$.

separate *Salmonella* transposon library of ~230,000 mutations identified 51 unique transposon integrations in the *spoT* gene (Table S1). The *spoT* transposon mutants in the library are viable, stable, and only experience mild negative selective pressure under laboratory conditions (29). Mapping of the sites of transposon integration into the *spoT* gene showed that the library did not contain any transposons in the HD- and SYN-encoded domains of *spoT* (Fig. 1A). However, the 3′-end 972 bases of *spoT* comprised a large number of transposon integration sites, consistent with the number expected for random integration. In contrast, the entire *relA* locus contained 161 Tn integrations spread throughout the gene (not shown). The lack of transposons in the 1,100 bases of the 5′ end of *spoT* encoding the SYN and HD catalytic domains suggests that such integrations render the dominant SpoT hydrolase activity nonfunctional, resulting in nonviable phenotypes in otherwise *relA*$^+$ bacteria. These data indicate that stable mutations can be generated in the C terminus of the *Salmonella* SpoT protein. The following investigations exploited this observation to molecularly characterize the contributions of *spoT* to *Salmonella* virulence.

SpoT plays unique roles in *Salmonella* virulence. One of the *spoT* transposon insertions mapped at base 1131 within the *spoT* gene, at the 5′-end of the TGS domain in the C-terminal domain (CTD) regulatory region, indicating that the region of 932 bases downstream was dispensable for survival *in vitro*. To better understand the biochemical characteristics of the SpoT variant lacking the regulatory C-terminal region, an in-frame stop codon was introduced at base 1131 followed by the *cat* gene (Fig. 1B), yielding the *spoT*-Δ*ctd* mutant that lacks all C-terminal regulatory domains. This strain was virulent in C57BL/6 mice but attenuated in C3H/HeN mice that express a functional natural resistance-associated macrophage protein (NRAMP) divalent cation transporter in phagosomal membranes (Fig. 3A). Analysis of the competitive index showed that the *spoT*-Δ*ctd* mutant is over 100,000 times more attenuated than wild-type controls in C3H/HeN mice 13 to 17 days after oral delivery (Fig. 3B; see Fig. S3 in the supplemental material). We have also found that Δ*relA* mutant *Salmonella* is attenuated in a C3H/HeN oral infection model (10). Collectively, these findings demonstrate that the *spoT* gene plays unique roles in *Salmonella* pathogenesis that cannot be performed by *relA* alone, and vice versa.

Capacity of the *spoT*-Δ*ctd* allele to metabolize (p)ppGpp. We next examined the ability of *spoT*-Δ*ctd* mutant *Salmonella* to synthesize and degrade (p)ppGpp. To induce (p)ppGpp synthesis, bacterial cultures were treated with serine hydroxamate (SHX), an irreversible inhibitor of seryl-tRNA synthetases that activates RelA in response to deacylated tRNAs erroneously loaded into the A site of the ribosome (30). In contrast to Δ*relA* isogenic controls, *Salmonella* expressing the *spoT*-Δ*ctd* allele produced as much (p)ppGpp after serine hydroxamate treatment as wild-type bacteria (Fig. 4A). These findings indicate that RelA is functional in *spoT*-Δ*ctd* mutant *Salmonella*. To examine the (p)ppGpp-hydrolytic activity associated with the *spoT*-Δ*ctd* allele, tetracy-

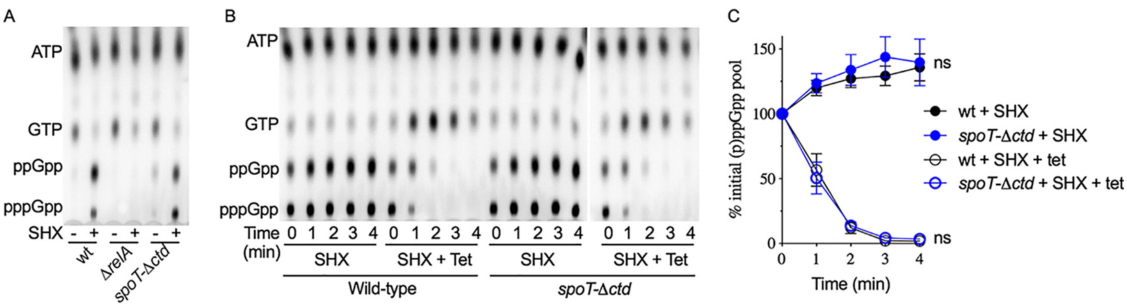

FIG 4 (p)ppGpp metabolism in *spoT-Δctd* mutant *Salmonella*. (A) Representative thin-layer chromatography (TLC) autoradiogram of ^{32}P-labeled nucleotide extracts from wild-type (wt), Δ*relA*, and *spoT-Δctd* mutant *Salmonella*. Where indicated, the cultures were treated with 0.4 mg/ml serine hydroxamate (SHX) for 3 min. (B) Hydrolysis of (p)ppGpp in wt and *spoT-Δctd* mutant *Salmonella* pretreated with 0.4 mg/ml SHX for 3 min before the addition of 70 μg/ml tetracycline for the indicated times. Each blot is representative of 3 or 4 independent experiments. (C) The % of ppGpp plus pppGpp after the addition of tetracycline was determined from autoradiograms in panel B. The data are expressed as mean ± standard deviation (SD) from 6 replicates; ns, not significant compared to wt controls.

cline was added to *Salmonella* pretreated with serine hydroxamate. Tetracycline binds to the A site of the ribosome, thereby not only blocking tRNA loading but also inhibiting RelA-mediated (p)ppGpp synthesis (13). Wild-type and *spoT-Δctd* mutant *Salmonella* treated with serine hydroxamate accumulated (p)ppGpp with similar kinetics, and the addition of tetracycline resulted in similar rates of (p)ppGpp hydrolysis (Fig. 4B). The estimated half-live of (p)ppGpp was <1 min in both strains (Fig. 4C). These findings suggest that the (p)ppGpp hydrolytic activity is fully functional in *spoT-Δctd* mutant *Salmonella*.

Growth of *spoT-Δctd* mutant *Salmonella* in minimal media. The growth of wild-type and *spoT-Δctd* mutant *Salmonella* was similar in LB broth, but growth of the *spoT-Δctd* mutant was slightly delayed when tested in E salts minimal medium supplemented with glucose and citric acid (Fig. 5A), suggesting that SpoT contributes to the growth of *Salmonella* in glucose and citric acid as the sole carbon sources. To examine the phenotype of *spoT-Δctd* mutant *Salmonella* during nutritional downshifts,

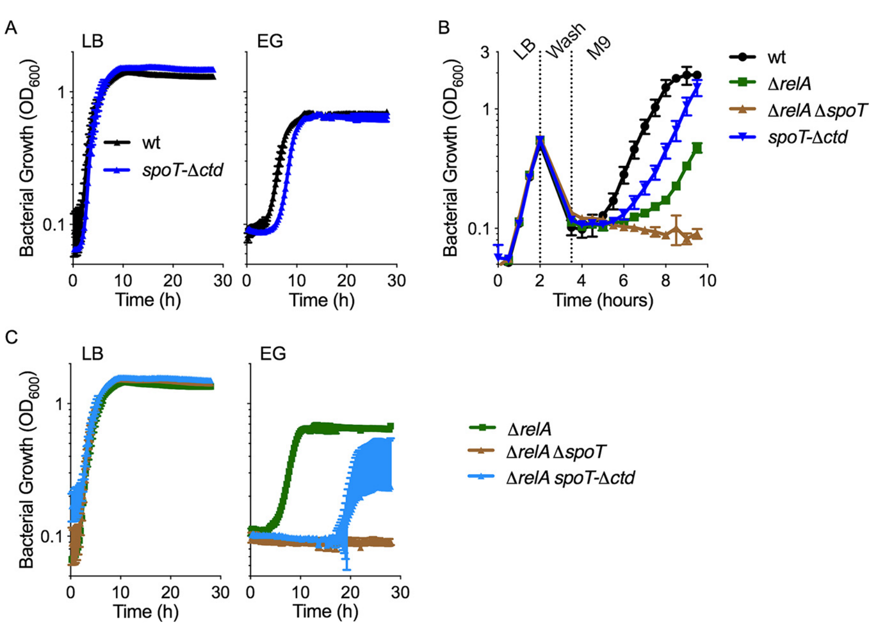

FIG 5 Growth of *spoT-Δctd* mutant *Salmonella* in medium. (A and C) Growth of the indicated *Salmonella* strains in LB broth or EG minimal medium ($N = 5$, mean) as measured by OD_{600} with a Bioscreen plate reader. (B) Recovery of *Salmonella* from nutrient downshift. Bacteria pregrown in LB broth were washed and resuspended in M9 minimal medium ($N = 3$, mean ± standard error of the mean [SEM]).

bacteria pregrown to the mid-exponential phase in LB broth were washed and transferred to M9 minimal medium containing glucose as the sole carbon source. Compared to wild-type controls, *spoT*-Δ*ctd* mutant *Salmonella* required slightly longer times to grow after this nutritional downshift. The growth defect of *spoT*-Δ*ctd* mutant *Salmonella* was less pronounced than that experienced by Δ*relA* mutant *Salmonella*. A Δ*relA* Δ*spoT* mutant *Salmonella* strain failed to grow in M9 minimal medium (Fig. 5B). These results confirm the importance of (p)ppGpp for growth during the transition to low-nutrient medium and strongly suggest that (p)ppGpp synthesized by both RelA and SpoT supports an efficient transition.

The growth defects exhibited by *spoT*-Δ*ctd* mutant *Salmonella* during a nutritional downshift suggest that the SpoT-Δctd variant is defective at producing (p)ppGpp under some conditions. To test this hypothesis further, the *spoT*-Δ*ctd* allele was transduced into a Δ*relA* mutant *Salmonella* background. The Δ*relA spoT*-Δ*ctd* double mutant grew as well as the Δ*relA* mutant controls in LB broth, but exhibited extreme growth delays in EG minimal medium (Fig. 5C). LB broth is rich in amino acids and short peptides, supporting robust growth of *Salmonella* independently of (p)ppGpp synthesis. EG minimal medium fails to sustain growth of Δ*relA* Δ*spoT* mutant *Salmonella* (Fig. 5C), because the inability of this strain to synthesize (p)ppGpp results in functional amino acid auxotrophies. The extreme growth defects of the Δ*relA spoT*-Δ*ctd* double mutant in EG minimal medium provide genetic proof of the defective (p)ppGpp synthetic capacity of the SpoT-Δctd enzyme.

Resistance of *spoT*-Δ*ctd* mutant *Salmonella* to oxidative and nitrosative stresses. Macrophages use reactive oxygen and nitrogen species generated by NADPH oxidase and inducible nitric oxide (NO) synthase to impede intracellular *Salmonella* replication (31–33). *Salmonella* counteract oxidative and nitrosative stress via an RelA-mediated stringent response (10, 28). We assessed whether *spoT* also contributes to the antioxidant and antinitrosative defenses of *Salmonella*. These studies showed that *spoT*-Δ*ctd* mutant *Salmonella* was as resistant as the wild-type bacterium to either nitric oxide or H_2O_2 (see Fig. S4A, S4B in the supplemental material). Collectively, these data suggest that the (p)ppGpp-synthetase activity of SpoT is dispensable for *Salmonella* resistance to reactive nitrogen and reactive oxygen species in culture.

***spoT*-Δ*ctd* mutant *Salmonella* replicates poorly in macrophages and exhibits defects in SPI-2 gene transcription.** *Salmonella* requires (p)ppGpp to replicate in macrophages; however, the source of (p)ppGpp remains unknown (26). Our initial transposon screen suggested that *spoT* transposon mutants are defective for intracellular replication in macrophages. Accordingly, *spoT*-Δ*ctd* mutant *Salmonella* exhibited poor growth in J774 cells compared to Δ*relA* or wild-type controls (Fig. 6A), suggesting that (p)ppGpp produced by SpoT is important for the intracellular replication of *Salmonella*. Notably, the Δ*relA* Δ*spoT* strain grew even more poorly than *spoT*-Δ*ctd* mutant *Salmonella* in macrophages, suggesting that RelA-derived (p)ppGpp can partially compensate for the lack of (p)ppGpp synthesis in *spoT*-Δ*ctd* mutant *Salmonella*.

The replication of intracellular *Salmonella* within phagocytes is greatly dependent on expression of SPI-2 genes encoding a type III secretion system (T3SS) that allows remodeling of the *Salmonella*-containing vesicle into an environment suitable for bacterial growth (34). Expression of the SPI-2 type III secretion system relies on the kinetic effects (p)ppGpp exerts on the stable open complex associated with the AT-rich discriminator region of the *ssrAB* locus (35). To examine SPI-2 gene transcription in *spoT*-Δ*ctd* mutant *Salmonella*, we followed the activation of an *sifA*::*lacZY* reporter fusion. Remarkably, *spoT*-Δ*ctd* mutant *Salmonella* expressed low levels ($P > 0.001$) of *sifA*::*lacZY* compared to wild-type or Δ*relA* mutant controls in response to a Mg^{2+} and pH downshift (Fig. 6B). Real-time PCR confirmed the poor expression of *sifA* in *spoT*-Δ*ctd* mutant *Salmonella* (Fig. 6C). Consistent with previous reports, Δ*relA* Δ*spoT* mutants were unable to induce expression of this β-galactosidase SPI-2 reporter (36).

Expression of *sifA* requires the SPI-2 type III secretion system master regulator SsrB (37). To examine whether the poor *sifA*::*lacZY* expression in *spoT*-Δ*ctd* mutant *Salmonella* could be due to inadequate SsrB expression, an *ssrB-FLAG* epitope-tagged allele

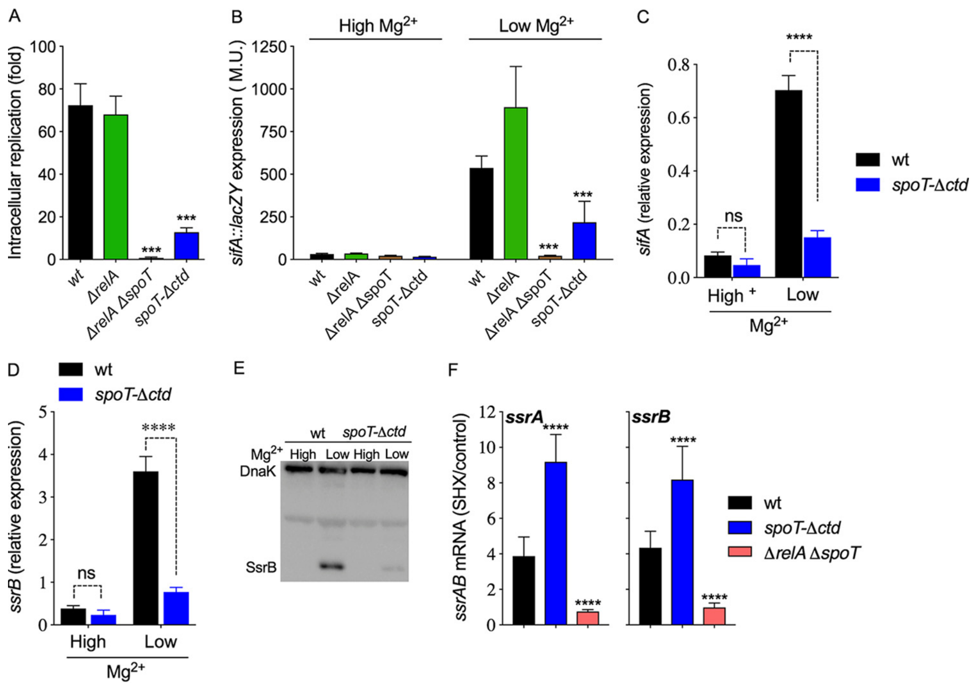

FIG 6 Intracellular growth and SPI-2 type III secretion system (T3SS) expression of *spoT-Δctd* mutant *Salmonella*. (A) Intracellular replication of *Salmonella* after 20 h of culture in J774 cells ($N = 12$, mean ± SEM; ***, $P < 0.001$ by one-way analysis of variance [ANOVA]). (B) Expression of the *sifA*::*lacZY* fusion in *Salmonella* cells grown in N9 minimal medium supplemented with high or low Mg^{2+} ($N = 3$, mean ± SEM; ***, $P < 0.001$ by two-way ANOVA). Transcription of *sifA* (C) and *ssrB* (D) mRNA in *Salmonella* cells grown in high- and low-Mg^{2+} N9 medium ($N = 6$; ****, $P < 0.0001$; ns, not significant by two-way ANOVA). (E) Western blot of DnaK and SsrB proteins in *Salmonella* experiencing a Mg^{2+} downshift in N9 minimal medium (representative of 6 independent blots). (F) Transcription of *ssrA* and *ssrB* in *Salmonella* grown in M9 media to an OD_{600} of 0.3. Some of the specimens were treated with 0.4 mg/ml of serine hydroxamate (SHX) for 30 min. The data are expressed as the ratio of SHX-treated cells over untreated controls ($N = 8$; ****, $P < 0.0001$ compared to wt controls by one-way ANOVA).

was transduced into *spoT-Δctd* mutant *Salmonella*. Compared to wild-type controls, *spoT-Δctd* mutant *Salmonella* exhibited defective transcription (Fig. 6D) and translation (Fig. 6E) of *ssrB* upon Mg^{2+} and pH downshifts. *Salmonella* expressing the *spoT-Δctd* allele was still capable of inducing *ssrA* and *ssrB* gene transcription if (p)ppGpp synthesis was stimulated with the RelA inducer serine hydroxamate (Fig. 6F). These data suggest that (p)ppGpp synthesis from SpoT optimizes SPI-2 type III gene transcription through expression of the *ssrB* master regulator, when stimulated with changes in magnesium and pH levels.

SpoT induces gene transcription in intracellular *Salmonella*. To determine whether SpoT contributes to SPI-2 gene expression in macrophages, transcription was studied in intracellular *Salmonella* isolated from J774 cells 8 h after infection. Consistent with the *in vitro* analysis, *spoT-Δctd* mutant *Salmonella* expressed lower levels of the *ssrB* gene encoding the SPI-2 master regulator (Fig. 7A). In addition, the *spoT-Δctd* strain expressed lower levels of *spiC*, *ssaV*, and *sifA*, which encode components of the SPI-2 secretion apparatus or effectors (Fig. 7A). Because the attenuation of *spoT-Δctd* mutant *Salmonella* was noted in C3H/HeN mice expressing a functional NRAMP locus, we tested whether (p)ppGpp synthesized by SpoT may also promote transcription of loci associated with cation uptake systems (Fig. 7B). The *znuA* gene, encoding a high-affinity zinc-binding periplasmic cassette, was expressed 5-fold less in *spoT-Δctd* mutant than in wild-type *Salmonella.* In contrast, the divergently regulated *znuC* component encoding a cytosolic ATPase was similarly expressed by wild-type and *spoT-Δctd* mutant *Salmonella.* In contrast to *mntH* and *mntP* loci encoding manganese uptake and homeostasis, the manganese uptake system encoded within the *sit* operon was expressed to lower levels in *spoT-Δctd* mutant *Salmonella* than in wild-type controls. We

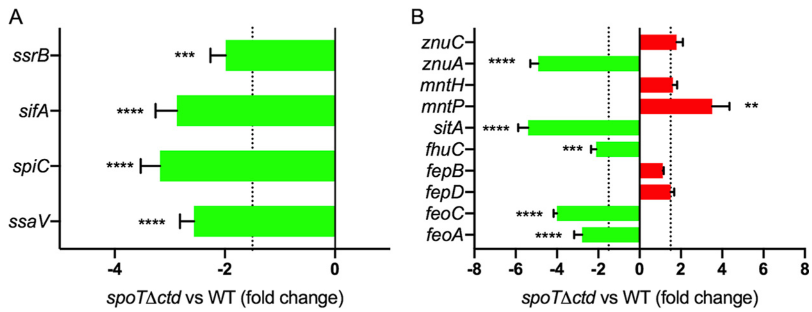

FIG 7 Intracellular expression of SPI-2 genes and divalent cation uptake systems. Quantitative real-time PCR (qRT-PCR)-based mRNA expression analyses illustrating the differential gene regulation of (A) SPI-2 genes and (B) metal cation uptake genes in *spoT*-Δ*ctd* mutant *Salmonella*. Green and red bars represent downregulated and upregulated genes compared to wild-type controls. The data are the mean ± SD from 3 or 4 independent experiments with 3 technical replicates each. The data were normalized using an average value of the *rpoD* gene. The vertical dotted line represents the 1.5-fold up- or downregulation considered to indicate a significant change. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (*spoT*-Δ*ctd* mutant strain compared to wild-type controls by one-way ANOVA).

found that SpoT is also needed for optimal expression of ferrous and ferric iron transport systems encoded by *feo* and *fhu* genes, but not for expression of *fepB* and *fepD*. The organization of these genes in the genome can be seen in Fig. S5 in the supplemental material. Together, these findings indicate that (p)ppGpp synthesized by SpoT activates transcription of both the SPI-2 genetic program and some metal cofactor acquisition systems in intracellular *Salmonella*.

SpoT activates gene transcription in response to the acidification of the phagosome. We modified the downshift growth conditions to gain more knowledge about the signals that activate the synthetase activity of SpoT intracellularly. *Salmonella* grown in N9 medium activated *sifA*::*lacZY* expression in a SpoT-dependent manner in response to acidification alone but did not significantly activate expression in response to a Mg^{2+} downshift alone (Fig. 8A). However, acidity and low Mg^{2+} concentrations synergized to generate a strong induction of *sifA*::*lacZY* transcription. These data suggest that SpoT synthetase activity is turned on in response to low pH. To test this idea, we monitored the production of (p)ppGpp in *Salmonella* grown in morpholinepropanesulfonic acid (MOPS)-glucose medium (pH 7 or 5.5) containing all amino acids (Fig. 8B). MOPS medium was chosen because the Casamino Acids present in N9 medium reduce the incorporation of ^{32}P into the nucleotide pool. In contrast to *spoT*-Δ*ctd* mutant *Salmonella*, wild-type controls produced ppGpp upon culture in MOPS-glucose medium (pH 5.5) for 5 min. However, the *spoT*-Δ*ctd Salmonella* strain produced as much ppGpp as wild-type controls when exposed to H_2O_2 (Fig. S5), which stimulates ppGpp synthesis through RelA (28). The intracellular growth advantage associated with the expression of full-length SpoT was lost in J774 cells treated with the vacuolar ATPase inhibitor bafilomycin (Fig. 8C). Bafilomycin treatment also dramatically reduced the expression of SPI-2 genes, *znuA*, *sitA*, and *feoC* in intracellular *Salmonella* (Fig. 8D). The inhibitory effects were more dramatic in wild-type *Salmonella* than in *spoT*-Δ*ctd* mutant controls. No transcriptional differences were noted between wild-type and *spoT*-Δ*ctd* mutant *Salmonella* treated with bafilomycin. Together, these investigations indicate that acidification of the phagosome activates (p)ppGpp production by SpoT, upregulating transcription of SPI-2 and metal cofactor acquisition systems that are needed for the intracellular fitness of *Salmonella*.

DISCUSSION

Despite the importance of the role (p)ppGpp plays in *Salmonella* virulence (26), the specific roles of the (p)ppGpp-synthetases RelA and SpoT have not been adequately characterized. The conditional essentiality of the *spoT* gene and its interrelationship with *relA* have made examination of SpoT function challenging. Here, we identified a SpoT variant with apparently normal hydrolase activity but defective (p)ppGpp syn-

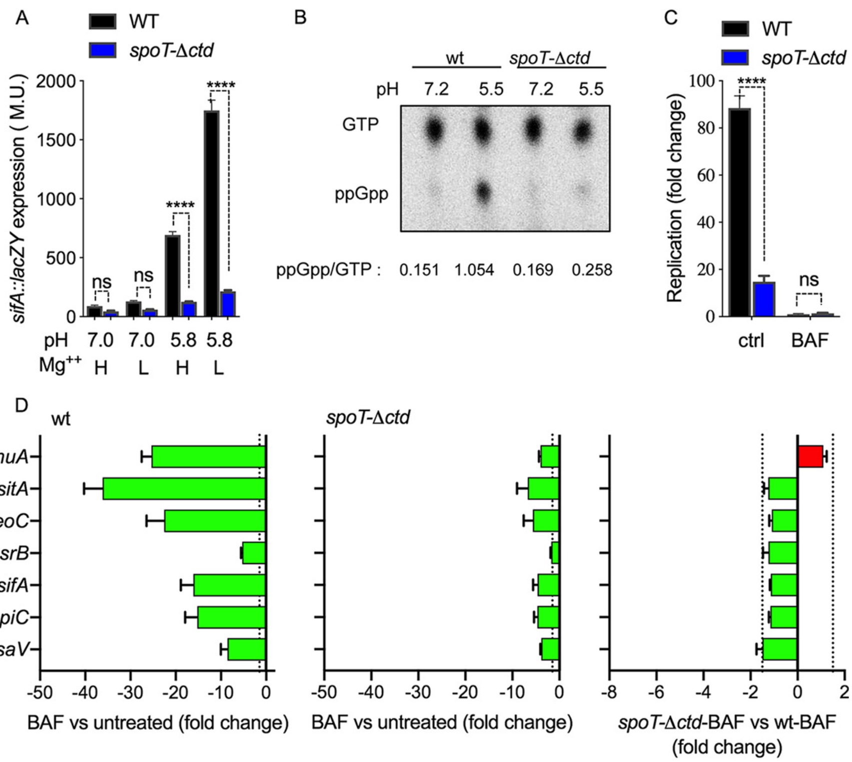

FIG 8 Sensing of phagosomal acidity activates SpoT-mediated gene transcription. (A) Expression of *sifA*::*lacZY* fusion in *Salmonella* grown in N9 minimal medium adjusted to pH 7.0 or 5.8. Where indicated, the cultures contained 10 mM (H) or 8 μM (L) $MgCl_2$. $N = 8$; ns, not significant; ****, $P < 0.0001$ by two-way ANOVA. (B) Detection of the TLC autoradiogram of [^{32}P]-labeled nucleotides from wild-type (wt) and *spoT*-Δ*ctd* mutant *Salmonella* after 5 min treatment at pH 5.5. (C) Effect of the ATPase proton inhibitor bafilomycin on the growth of *Salmonella* in J774 cells. Some of the cultures were treated with 100 nM bafilomycin for 20 h before enumeration of intracellular bacterial burden. $N = 12$; ns, not significant; ****, $P < 0.0001$ by two-way ANOVA. (D) qRT-PCR-based intracellular expression of SPI-2 and metal cation uptake genes in wild-type and *spoT*-Δ*ctd* mutant *Salmonella*. J774 cells infected with *Salmonella* were treated for 8 h with 100 nM the ATPase inhibitor bafilomycin. Green and red bars represent downregulated and upregulated genes. The data are the mean ± SD from 3 or 4 independent experiments with 3 technical replicates each. The data were normalized using an average value of the *rpoD* gene. The vertical dotted line represents the 1.5-fold up- or downregulation considered to exhibit a significant change. Bafilomycin significantly downregulated all of the genes assayed in wt and *spoT*-Δ*ctd* mutant *Salmonella*, as measured by one-way ANOVA. No differences were found with bafilomycin treatment between wt and *spoT*-Δ*ctd* mutant *Salmonella*.

thetic activity. The latter feature of the *spoT*-Δ*ctd* allele is phenotypically analogous to the SpoT SYN point mutants described in *E. coli* (38), making the SpoT-Δctd variant a potentially powerful tool for further studying the biology of (p)ppGpp synthesized by SpoT.

The *Salmonella spoT*-Δ*ctd* mutant is attenuated in a model of acute nontyphoidal *Salmonella* infection and grows poorly in phagocytic cells, indicating that (p)ppGpp synthesized by SpoT supports *Salmonella* pathogenesis (Fig. 9). Specifically, *spoT*-Δ*ctd* mutant *Salmonella* expresses low levels of *ssrA* and *ssrB*, which encode the master regulatory system that controls global SPI-2 gene transcription. Guanosine tetraphosphate promotes SPI-2 expression by relieving the kinetic constrains the AT-rich region of the *ssrA* promoter imposes on RNA polymerase (35). Defective expression of SPI-2 genes is likely to contribute to the intracellular growth defect and attenuation of *spoT*-Δ*ctd* mutant *Salmonella.* In addition, a functional SpoT activates transcription of iron, manganese, and zinc uptake systems in response to acidification of the phagosome. Given the attenuation of *spoT*-Δ*ctd* mutant *Salmonella* in C3H/HeN mice expressing a functional NRAMP locus that has recently been implicated in the uptake of magnesium (39), it is also possible that ppGpp synthesized by SpoT play roles in the activation of magnesium uptake systems. Together, our investigations identify SpoT as

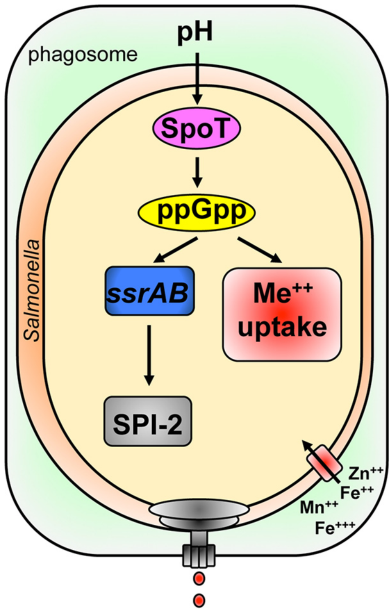

FIG 9 Model of regulation of *Salmonella* virulence programs by ppGpp synthesized by SpoT. Low phagosomal pH induces synthesis of (p)ppGpp by the bifunctional SpoT protein. Production of this nucleotide alarmone induces transcription of the *ssrAB* locus that encodes the master SPI-2 two-component regulatory system. SsrA and SsrB activate expression of the SPI-2 genes encoding a type III secretion system that delivers effector proteins into eukaryotic cells. In addition, the (p)ppGpp produced by SpoT activates expression of manganese, zinc, and iron uptake systems, further contributing to *Salmonella* virulence.

a critical regulator of intramacrophage virulence. It remains likely that (p)ppGpp synthesized by SpoT in response to the acidification of the phagosome regulates global gene expression in intracellular *Salmonella*.

Our studies (here and in reference 10) indicate that (p)ppGpp synthesized by either RelA or SpoT can be sufficient for some aspects of *Salmonella* pathogenesis, including the survival in C57BL/6 mice. However, (p)ppGpp synthesized by either RelA or SpoT each supports unique aspects of *Salmonella* pathogenesis. Our recent work has demonstrated that RelA is required for resistance to nitrosative stress (10). NO induces functional auxotrophies for branch chain amino acids, eliciting (p)ppGpp synthesis by RelA near the A site of the ribosome (10). Here, we reported that *spoT*-Δ*ctd* mutant *Salmonella* express low levels of SPI-2 genes and SsrB protein in response to acidification, a relevant signal encountered by *Salmonella* in the phagosome of macrophages. A specific role for SpoT, but not for RelA, has also been documented in the adaptation of *Legionella* to its macrophage vacuole (40). However, the role played by SpoT in *Legionella* pathogenesis was mapped to its hydrolytic activity. It remains unknown if the hydrolytic activity of SpoT contributes to *Salmonella* pathogenesis.

Our results demonstrate that SpoT synthesizes (p)ppGpp in response to acidic pH. The other effector of the stringent response, the transcriptional regulator DksA, changes its activity as the pH decreases (41). It is not clear if SpoT can similarly sense changes in cytosolic pH. Fatty acid starvation leads to SpoT-mediated (p)ppGpp synthesis through the direct interaction of acyl carrier protein and the SpoT TGS domain. It is possible that sensing of acid pH by SpoT occurs indirectly through binding a protein partner. Future studies are required to examine exactly how phagosomal acidification mediates SpoT-derived (p)ppGpp synthesis in *Salmonella*.

Guanosine tetraphosphate generated by RelA or SpoT can regulate some aspects of *Salmonella* virulence. However, there also seems to be a specialized division of labor among RelA and SpoT according to signals encountered during colonization and infection of the mammalian host (here and in reference 10).

MATERIALS AND METHODS

Defined minimal media. EG (57.4 mM K_2HPO_4, 1.7 mM $MgSO_4$, 9.5 mM citric acid, 16.7 mM H_5NNaPO_4, 0.4% D-glucose; pH 7.0 unless indicated otherwise), M9 (48 mM $Na_2HPO_4 \cdot 7H_2O$, 22 mM KH_2PO_4, 8.56 mM NaCl, 18.69 mM NH_4Cl, 1 mM $MgSO_4$, 100 μM $CaCl_2$, 0.2% D-glucose, and 50 μM $FeSO_4 \cdot 7H_2O$; pH 7.1), N9–high Mg^{2+} [100 mM Tris-HCl, 5 mM KCl, 7.5 mM $(NH_4)SO_4$, 1 mM KH_2PO_4, 38 mM glycerol, 0.1% Casamino Acids, and 10 mM $MgCl_2$; pH 8.0], N9–low Mg^{2+} [100 mM Tris-HCl, 5 mM KCl, 7.5 mM $(NH_4)SO_4$, 1 mM KH_2PO_4, 38 mM glycerol, 0.1% Casamino Acids, 8 μM $MgCl_2$; pH 5.8), and MOPS [40 mM MOPS buffer, 4 mM tricine, 0.4% D-glucose, 40 μg/ml of each amino acid except serine, 2 mM K_2HPO_4, 10 μM $FeSO_4 \cdot 7H_2O$, 9.5 mM NH_4Cl, 276 μM K_2SO_4, 500 nM $CaCl_2$, 50 mM NaCl, 525 μM $MgCl_2$, 2.9 nM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 400 nM H_3BO_3, 30 nM $CoCl_2$, 9.6 nM $CuSO_4$, 80.8 nM $MnCl_2$, and 9.74 nM $ZnSO_4$; pH 7.2] minimal media were used in the course of these investigations (42).

Bacterial strains and genetics. *Salmonella enterica* serovar Typhimurium 14028s and its mutant derivatives were maintained on LB broth (Lennox). Bacterial cultures were grown at 37°C in a shaking incubator. Gene deletions were performed with the λ Red recombinase system as previously described (43, 44) with minor modifications. Specifically, the *cat* gene was PCR amplified from pKD3 with the primers GCTACAACAGAGCGCCGGTAGTTCGTTTGAATTTATCGAATAATATGAATATCCTCCTTAGTT and CAT TTCGCATATACGCGCATAACGTTTTGGATTCATAGCGGTGTAGGCTGGAGCTGCTTC. This PCR product was recombined after 1,131 bp of *spoT*, replacing the last 981 bp of the gene with an in-frame stop codon and the *cat* gene, generating the *spoT*-Δ*ctd* allele. PCR products were transformed into wild-type *Salmonella* carrying the pTP233 plasmid encoding the λ Red recombinase. Transformants were confirmed by PCR. Table S2 lists the strains used in this study.

Generation and analyses of *Salmonella* transposon libraries. The libraries used here were generated as previously described (29). Briefly, EZ-Tn*5* <KAN-2> (Lucigen) was modified to introduce an N_{18} barcode adjacent to an Illumina read 1 sequence. A library of over 230,000 different insertion mutants was constructed by mixing transposase and barcoded construct and by subsequent electroporation into electrocompetent *Salmonella*. The barcode associated with each unique Tn*5* insertion position was determined by Illumina sequencing of PCR-amplified flanking regions.

DNA library preparation, sequencing and data analysis. The methods for DNA library preparation, sequencing, and data analysis were previously described (29, 45). In brief, bacteria were recovered and grown in LB + 60 μg/ml kanamycin. Bacteria were pelleted, lysed, and subjected to PCR using primers directly flanking the N_{18} barcode. The frequency of each barcode was enumerated by Illumina sequencing of 20 bases. The aggregated abundances for the input and output libraries were statistically analyzed using DESeq2, and the $\log_2$ fold change, and false-discovery rates (FDRs) were reported.

ClueGO analysis. Transposon mutants that were negatively selected in the output population (adjusted *P* value of less than 0.05 compared to the input), and that were contained within the coding region for the gene they interrupted, were analyzed via pathway analysis with ClueGO (46, 47) version 2.5.4 in Cytoscape (48, 49) version 3.7.1. The following *S.* Typhimurium 14028s ontologies were referenced in the pathway analysis: KEGG_05.03.2019, GO_BiologicalProcess_10.02.2015_20h06, and GO_MolecularFunction_10.02.2015_20h06. Only pathways that had at least 10% of genes represented and a Bonferroni-corrected right-sided hypergeometric test with mid-*P* values less than or equal to 0.05 were displayed. Gene ontology (GO) levels 3 to 10 were used. Larger nodes represent more significant terms, while node shading is proportional to the percentage of genes represented for that term. Grouping of nodes was done with a perfuse directed layout based on a kappa score of 0.67. Group terms are based on the node with the highest percentage of genes per term compared to the cluster. Any groups sharing 40% of genes or 30% of terms were merged.

Growth assays. Overnight bacterial cultures grown in LB broth were diluted 1:1,250 into either fresh LB broth or EG minimal medium. The optical densities at 600 nm (OD_{600}) of 200-μl aliquots of bacterial cultures were recorded with a Bioscreen C plate reader (Growth Curves USA, Piscataway, NJ) every 15 min for 40 h. The time (h) at which half of the maximal OD_{600} was reached for each culture was calculated by exponential regression.

Nutrient downshift assay. *Salmonella* cells grown to the mid-exponential phase (OD_{600}, 0.4 to 0.6) in LB broth were collected, washed 3× with M9 minimal medium, and resuspended in M9 minimal medium (OD_{600}, ~0.1). Growth was tracked by measuring OD_{600}.

NO recovery assays. *Salmonella* cultures grown to the early exponential phase (OD_{600}, 0.2 to 0.3) in M9 minimal medium were challenged with 750 μM spermine NONOate. OD_{600} measurements were recorded every 30 min for 4.5 h. *Salmonella* cultures were grown to the early exponential phase (OD_{600}, 0.2 to 0.3) in EG minimal medium supplemented with Casamino acids (EGCA) and were challenged with 400 μM of H_2O_2. OD_{600} measurements were recorded every 30 min for 4.5 h.

H_2O_2 killing. LB overnight cultures of *Salmonella* were diluted to approximately 10^6 CFU/ml in phosphate-buffered saline (PBS). Cultures were challenged with 100 μM of H_2O_2 for 2 h at 37°C and 5% CO_2. The percent survival was calculated compared to time zero before H_2O_2 challenge.

Measurement of (p)ppGpp pools. (p)ppGpp pools were visualized as previously described (10, 50). *Salmonella* was grown in MOPS minimal medium supplemented with 0.4% D-glucose, 40 μg/ml of each amino acid except serine, and low-concentration phosphate (0.4 mM). *Salmonella* cultures grown to an OD_{600} of 0.2 were labeled with 10 μCi/ml of phosphorus-32 (^{32}P; PerkinElmer, Waltham, Massachusetts) for approximately 1.5 doubling times. Bacteria were treated with 0.4 mg/ml serine hydroxamate and 70 μg/ml tetracycline. For the acid challenge studies, the pH of the medium was adjusted to 5.5 with 1 M HCl. Aliquots of bacterial cultures (200 or 400 μl) were added to 80 or 160 μl of ice-cold 50% formic acid, respectively. Samples were thoroughly mixed and incubated on ice for at least 20 min. Samples were centrifuged at 13,000 rpm for 5 min and then returned to ice. Formic acid extracts (5 μl) were spotted along the bottom of polyethyleneimine (PEI)-cellulose thin-layer chromatography (TLC) plates (Millipore, Darmstadt, Germany). Samples were separated using 1.25 M KH_2PO_4 (pH 3.4) as a solvent system. TLC plates were air dried, wrapped in plastic, and exposed against K-screens overnight. K-screens were visualized using a phosphorimager (Bio-Rad, Hercules, California).

β-Galactosidase assays. SPI-2 gene expression was performed as previously described (51). Briefly, overnight LB broth cultures of *Salmonella* expressing the *sifA*::*lacZY*::Km single-copy chromosomal fusion (51) were diluted 1:100 into N9–high Mg^{2+} minimal medium and were grown for 3.5 to 4.5 h to an OD_{600} of approximately 0.5. Cultures were split, collected, washed three times with either N9–high Mg^{2+} or N9–low Mg^{2+} minimal medium, and resuspended in either N9–high Mg^{2+} or N9–low Mg^{2+} minimal medium at an OD_{600} of approximately 0.25. Cultures were grown for 3 h. The final culture OD_{600} was recorded, and 100 μl of culture was diluted into 900 μl of Z buffer with 3.9 μl of β-mercaptoethanol, 25 μl of 0.1% SDS, and 50 μl of chloroform. Specimens were mixed, and the reactions were started upon the addition of 200 μl of 4 mg/ml ortho-nitrophenyl-β-galactosidase. Reactions were stopped with the addition of 0.5 ml 1 M sodium carbonate. The A_{420} and A_{550} of 200 μl of each reaction were recorded, and Miller units were calculated according to the following equation: $[(A_{420} - \text{blank}) - 1.75 \times (A_{550} - \text{blank})]/[\text{time of reaction (min)} \times \text{volume of culture (ml)} \times (OD_{600}\ \text{culture} - \text{blank})]$.

Western blotting. Wild-type and *spoT*-Δ*ctd* mutant *Salmonella* expressing the *ssrB-FLAG*::Km chromosomal single-copy epitope-tagged allele were grown exactly as described above in the SPI-2 β-galactosidase assay. After 3 h of Mg^{2+} downshift, 1 ml of culture was mixed with 150 μl ice-cold 100% trichloroacetic acid and incubated on ice for 15 min. Specimens were collected at 16,000 $\times$ *g* for 10 min at 4°C, and the pellets were resuspended in PBS with 50 mM NaOH and 6$\times$ Laemmli buffer and boiled for 10 min. Approximately 50 ng of protein for each sample was loaded into a 12% polyacrylamide SDS gel and was electrophoresed at 80 V for 15 min in SDS running buffer (25 mM Tris base, 192 mM glycine, and 0.1% sodium dodecyl sulfate). Once the samples had entered the separating gel, the current was increased to 110 V for approximately 80 min. Samples were transferred to nitrocellulose membranes at 25 V for 30 min on a Bio-Rad semidry transfer apparatus with transfer buffer (48 mM Tris base, 39 mM glycine, 20% methanol [vol/vol]). Membranes were blocked in 5% skim milk in TBST (20 mM Tris base, 150 mM NaCl, pH 7.6, and 0.025% Triton X-100 [vol/vol]), gently rocking for 2 h at room temperature. Membranes were probed with primary anti-DnaK and anti-FLAG murine antibodies diluted 1:2,000 and 1:500, respectively, in 5% skim milk TBST for 2 h at room temperature. Membranes washed 3$\times$ with TBST buffer were probed at room temperature for 1 h with goat anti-mouse antibodies conjugated to horseradish peroxidase diluted 1:10,000 in 5% skim milk TBST. Membranes were then washed 3$\times$ with TBST buffer. The blots were processed using the ECL Prime Western blotting detection reagent (GE Healthcare, Chicago, IL) mixed as directed and added to the membranes (approximately 1 ml per blot); excess liquid was wicked away, and chemiluminescence was visualized with a gel dock (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

Transcriptional analysis of intracellular *Salmonella*. J774 macrophage-like cells were seeded on the 150-mm plate to 90% confluence. The cells were washed once with prewarmed PBS and culture in fresh RPMI medium. The macrophages were infected with overnight cultures of *Salmonella* at a multiplicity of infection (MOI) of 50. After 30 min, cells were washed with prewarmed PBS and cultured with fresh RPMI medium. After 30 min, 50 μg/ml gentamicin was added for 60 min, after which time the medium was replaced with fresh RPMI medium containing 10 μg/ml gentamicin. Where indicated, the cell cultures were treated with 100 nM bafilomycin. The cells were washed once with prewarmed PBS 8 h after infection and lysed in ice-cold PBS containing 0.1% Triton X-100. The cells were scraped, and the specimens were vortexed for 20 sec. Cell host debris was discarded by centrifugation at 800 to 1,000 rpm for 5 min, and bacteria present in the supernatants were collected by centrifugation at 8,000 rpm for 5 min. Bacterial pellets were collected for RNA isolation by centrifugation at 8,000 rpm for 5 min after a final wash in ice-cold PBS.

DNA-free RNA was obtained using a High Pure RNA isolation kit (Roche, Germany) according to the manufacturer's instructions. First-strand cDNA generation from total RNA was generated using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, USA). Relative mRNA quantitation was done using the SYBR green quantitative real-time PCR (qRT-PCR) master mix (Roche, Germany) using the primers described in Table S3 in the supplemental material. Data evaluation of 3 or 4 biological replicates done in triplicate was performed using the threshold cycle ($2^{-\Delta\Delta CT}$) method. Genes that exhibited >2-fold up- or downregulation were considered to exhibit a significant change.

Intracellular replication. Stationary-phase *Salmonella* cultures diluted in RPMI+ medium were added to 10^5 J774 cells at an MOI of 2. Plates were centrifuged for 2 min at 4,000 rpm and returned to a 37°C CO_2 incubator for 30 min. The *Salmonella*-containing RPMI+ medium was replaced with RPMI+ medium supplemented with 50 μg/ml gentamicin for 1 h and then replaced again with RPMI+ medium supplemented with 10 μg/ml gentamicin for either 1 or 19 additional hours. J774 cells were lysed with 0.1% Triton X-100 in PBS. Lysates were diluted and plated for CFU enumeration. To calculate fold replication, the number of intracellular *Salmonella* CFU recovered after 20 h of culture was normalized to the number of *Salmonella* CFU recovered after 2 h of infection.

Murine survival of infection. Mice were bred and maintained at the University of Colorado School of Medicine according to IACUC protocols. C57BL/6 or C3H/HeN mice (6 to 8 weeks old) were infected orally (p.o.) with 5×10^6 or 10^7 CFU of *Salmonella*, respectively. Survival of mice after infection was monitored for 28 days. Mice showing signs of disease were humanly euthanized by CO_2 inhalation and cervical dislocation. In addition, some C3H/HeN mice were inoculated p.o. with 10^7 CFU containing equal numbers of wild-type and *spoT*-Δ*ctd* mutant *Salmonella*. The mice were euthanized between 7 and 9 days after infection, when signs of disease first appeared. Bacterial burden was quantified in livers and spleens, and the competitive index was assessed as described (52).

Statistical analysis. Determination of statistical significance between two comparisons was achieved using an unpaired *t* test. Determination of statistical significance between multiple comparisons was done using a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison posttest. Statistical significance for mouse survival curves was determined using the log rank test. A *P* value of $<$0.05 was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, DOCX file, 1.1 MB.
FIG S2, DOCX file, 1.2 MB.
FIG S3, DOCX file, 0.2 MB.
FIG S4, DOCX file, 0.2 MB.
FIG S5, DOCX file, 0.7 MB.
FIG S6, DOCX file, 0.3 MB.
TABLE S1, DOCX file, 0.1 MB.
TABLE S2, DOCX file, 0.05 MB.
TABLE S3, DOCX file, 0.1 MB.

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